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KINETICS AND MECHANISMS OF TRYPSIN- AND THROMBIN-CATALYSED AND RELATED REACTIONS

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ABSTRACT

In Part I, confirmatory evidence has been adduced for the mechanism of lactamization of methyl esters of α-N-toluene-p-sulphonyl derivatives of αω-diamino-acids in aqueous solution, which was described in the Final Technical Report under Contract DA-91-591-EUC-1301. Participation of the sulphonamide-group has been excluded, since the kinetics of lactamization of methyl γ-amino-L-α-N-methyltoluene-p-sulphonamidobutyrate (AMTBME), in which ionization of the sulphonamide group is precluded, are exactly parallel to those of the compounds studied earlier.

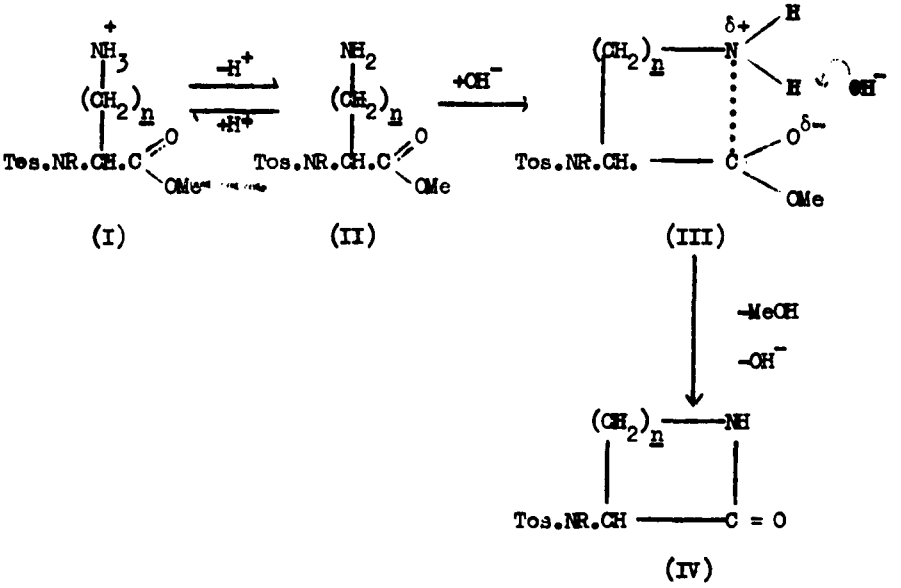
In Part II, kinetic studies of the action of trypsin on various synthetic substrates have been amplified. The significance of the experimentally observed values of various kinetic constants is considered theoretically in the light of the probable three-step mechanism for catalysis by proteolytic enzymes such as trypsin. In the trypsin-catalysed hydrolysis of esters of α-N-toluene-p-sulphonyl-L-arginine, acylation and deacylation both appear to influence the overall rate of reaction, while in the case of esters of α-N-benzoyl-L-arginine, deacylation is rate-determining. An ionizable group, whose apparent pK_a and heat of ionization are consistent with those of an imidazole nucleus, is implicated in the trypsin-catalysed hydrolysis of α-N-toluene-p-sulphonyl-L-arginine methyl ester. Cholate ion accelerates the trypsin-catalysed hydrolysis of esters of α-N-toluene-p-sulphonyl-L-arginine and -homoarginine slightly inhibits the hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE). Using substrate concentrations high enough to ensure obedience to zero-order kinetics, activation constants have been determined. Those for the trypsin-catalysed hydrolysis of the methyl and ethyl esters of α-N-toluene-p-sulphonyl-L-arginine (TAME and TAEE) converge to practically identical values with increasing cholate-concentration. This suggests that in presence of sufficient cholate the acylation step is sufficiently accelerated so that deacylation of a common intermediate becomes rate-determining. The same effect is possibly exerted by cholate on the hydrolysis of α-N-toluene-p-sulphonyl-L-homoarginine methyl ester, and supporting evidence is adduced from determination of apparent K_M values. α-N-Toluene-p-sulphonyl-L-ornithine methyl ester is slowly hydrolysed by trypsin at pH 7.0 and the kinetics are first-order with respect to substrate. There is evidence that this reaction is not completely stereospecific. The hydrolysis is appreciably accelerated by cholate with an attendent decrease in the apparent energy of activation for the reaction.

In Part III, we report a study of the kinetics of thrombin-catalysed reactions. Using the Ehrenpreis and Scheraga unit (J. Biol. Chem., 1957, 227, 1043) as a means of expressing thrombin activity, it has been shown that the velocity of a thrombin-catalysed reaction is proportional to enzyme concentration. In contrast to

trypsin-catalysed reactions, the velocity of hydrolysis of TAME by thrombin is decreased by sodium or potassium chloride. Like trypsin-catalysed reactions, on the other hand, the rate-determining step for the hydrolysis of esters of α-*N*-benzoyl-*L*-arginine by thrombin appears to be deacylation. In the case of esters of α-*N*-toluene-*p*-sulphonyl-*L*-arginine, both acylation and deacylation influence the overall rate of hydrolysis. The methyl ester of α-*N*-toluene-*p*-sulphonyl-*L*-lysine (TLME) is hydrolysed by thrombin at a rate similar to that of TAME. Rate constants for the thrombin-catalysed hydrolysis of TAME and BAEE do not obey the Arrhenius law. The apparent dissociation constant of the ionizable group, which is involved in the thrombin-catalysed hydrolysis of TAME, is rather lower than that associated with the trypsin-catalysed hydrolysis of this substrate. Cholate and glycocholate accelerate the thrombin-catalysed hydrolyses of substrates containing the α-*N*-toluene-*p*-sulphonamide group, but inhibit the hydrolysis of BAEE. The apparent K_M values for the TAME- and BAEE-thrombin systems are both decreased by bile salts. The mechanism by which bile salts modify the kinetics of thrombin-catalysed reactions is probably similar to that postulated for trypsin-catalysed processes. Non-ionic detergents such as Tween 20, Tween 40 and Tween 80 accelerate the hydrolysis of both TAME and BAEE by thrombin.

PART I. THE KINETICS OF LACTAMIZATION OF METHYL ESTERS OF SOME α-N-TOLUENE-p-SULPHONYL DERIVATIVES OF αω-DIAMINO-ACIDS IN AQUEOUS SOLUTION.

In Part I of the Final Technical Report under the preceding contract (No. DA-91-591-EUC-1301), it was shown that lactamization of the methyl esters of α-N-toluene-p-sulphonyl-L-ornithine (TOME) and γ-amino-α-toluene-p-sulphonamidobutyric acid (ATBME) proceeded with hydroxyl-ion catalysis in the pH range 7.8 - 8.5. We proposed the following mechanism for this reaction:



(I) (II) (III) (IV)

The initial rate of proton release at constant pH, u_o, was given by the equation:

$$\frac{1}{\sqrt{u_o}} = \frac{[H^+]}{\sqrt{kKK_W[S]_o}} + \sqrt{\frac{K}{kK_W[S]_o}} \quad \ldots\ldots\ldots\ldots (1)$$

where k is the second-order rate constant for the lactamization process, K is the dissociation constant of the protonated amino-group of the ester (I), K_W is the ionic product of water, and $[S]_o$ is the initial concentration of substrate. The value of K, as evaluated from the application of equation (1) to our experimental data, was inconsistent with the participation of the sulphonamide group, and such a mechanism appeared unlikely. In order to obtain definitive evidence on this point, we have now synthesised methyl γ-amino-L-α-N-methyltoluene-p-sulphonamidobutyrate (AMTBME) in which ionization of the sulphonamide group is precluded. This compound was readily obtained by methylation of γ-benzyloxycarbonylamido-L-α-toluene-p-sulphonamidobutyric acid in a manner similar to that described for the synthesis of sarcosine (Cocker and Lapworth, J. Chem. Soc., 1931, 1894; Cocker, J. Chem. Soc., 1937, 1693). Hydrogenation and esterification completed the synthesis. The γ-benzyloxycarbonylamido-group was proved to be unaffected by methylation, since the acid afforded by hydrogenation yielded nitrogen when treated with nitrous acid in

a Van Slyke apparatus. In addition, the ultraviolet spectrum of the N-methyl acid, like that of N-toluene-p-sulphonylsarcosine, was identical in acid and alkaline solution, whereas that of γ-amino-L-α-toluene-p-sulphonamidobutyric acid exhibited a pronounced hyperchromic effect at high pH values resulting from ionization of the sulphonamide group. The kinetics of lactamization of AMTBME were found to be very similar to those of the two substrates previously studied, and our earlier views concerning the mechanism of lactamization are fully confirmed. Lactamization of these esters appears not to be subject to general-base catalysis, since neither pyridine nor imidazole affected the velocity of reaction. More kinetic runs have been carried out on TOME and ATBME since the last Final Technical Report, and the complete results are given in Table 1 and 2.

TABLE 1.

Initial rates ($10^3 v_o$, mole $l.^{-1} min.^{-1}$) of proton release during lactamization of the esters (II) (standard errors of the means in parentheses).

	R = H, n = 2			R = Me, n = 2	R = H, n = 3	
pH	20°	25°	30°	25°	25°	30°
7.80	0.76(0.01)	1.30(0.03)	4.47(0.04)	1.02(0.01)	1.72(0.05)	5.25(0.11)
7.90	1.22(0.06)	2.31(0.08)	6.62(0.17)	1.45(0.07)	2.98(0.07)	6.94(0.12)
8.00	1.86(0.03)	3.30(0.07)	8.80(0.22)	2.19(0.14)	4.32(0.17)	11.08(0.25)
8.10	2.53(0.04)	4.35(0.07)	11.34(0.41)	3.54(0.08)	6.27(0.08)	16.22(0.16)
8.20	3.75(0.07)	5.31(0.12)	15.89(0.69)	4.70(0.17)	9.30(0.40)	23.09(0.[illegible])
8.30	4.89(0.17)	7.58(0.24)	24.45(0.33)	6.18(0.44)	12.07(0.39)	30.35(0.77)
8.40	7.23(0.20)	9.48(0.36)	27.94(0.93)	8.27(0.53)	21.39(0.80)	44.44(1.32)
8.50	9.98(0.49)	12.70(0.19)	36.45(0.77)	11.32(0.28)	30.43(1.07)	59.77(4.19)

TABLE 2.

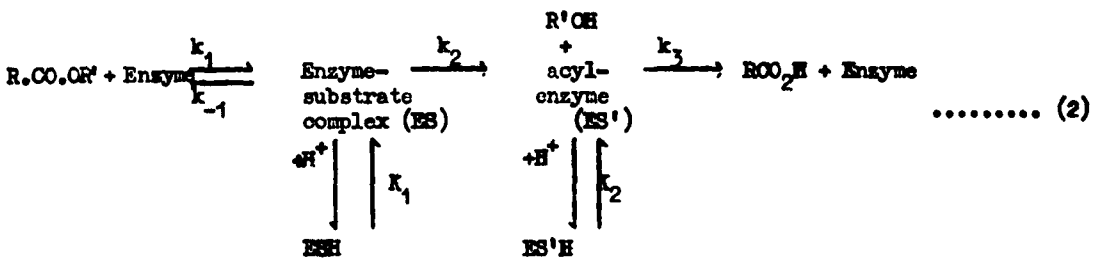
pK_a' values for the esters (II) and second-order rate constants ($10^{-4}k$, $mole^{-1} l. min.^{-1}$) for their lactamization (standard deviations in parentheses).

	R = H, n = 2			R = Me, n = 2	R = H, n = 3	
	20°	25°	30°	25°	25°	30°
pK_a'.....	8.72(0.10)	8.47(0.09)	8.42(0.03)	8.66(0.06)	8.97(0.20)	8.69(0.10)
$10^{-4}k$...	1.77(0.18)	1.54(0.13)	3.00(0.09)	1.45(0.09)	4.66(0.93)	5.13(0.52)

PART II. STUDIES OF THE KINETICS AND MECHANISM OF TRYPSIN-CATALYSED HYDROLYSES OF SYNTHETIC SUBSTRATES.

In Parts II and III of the Final Technical Report under contract DA-91-591-EUC-1301, we reviewed the available evidence related to the mechanism of action of trypsin and we presented the results of our kinetic studies using various synthetic substrates. We reported also that the trypsin-catalysed hydrolyses of the methyl esters of α-N-toluene-p-sulphonyl-L-arginine and -L-homoarginine (TAME and THAME respectively) were accelerated in the presence of cholate ions. We do not propose to review the literature again at length here, and we shall confine ourselves to presenting the results of our continued studies of trypsin mentioning only those publications which are pertinent to the development of our concept of the mechanism of action of trypsin.

Schwert and Eisenberg (J. Biol. Chem., 1949, 179, 665) found that, at substrate concentrations high enough to ensure that zero-order kinetics were obeyed, several esters of α-N-benzoyl-L-arginine were hydrolysed by trypsin at identical rates. The inference to be drawn from these experiments is that a common intermediate, (α-N-benzoyl-L-arginyl) trypsin, is formed and that the deacylation of this is rate-determining. The hydroxyl group in the serine residue at the active centre is probably the site of acylation. Strong evidence for such intermediates has been obtained in the case of chymotrypsin-catalysed reactions (P. Desnuelle in "The Enzymes", edited by P. D. Boyer, H. Lardy, and K. Myrbäck, Academic Press, N.Y. 1960, Vol. 4, p.93). The formation of cinnamoyl-chymotrypsin as an intermediate in the hydrolysis of esters of cinnamic acid by chymotrypsin has been particularly firmly established (Bender & Zerner, J. Amer. Chem. Soc., 1961, 83, 2391). The hydrolysis of N-trans-cinnamoylimidazole is catalysed by either trypsin or chymotrypsin at pH 5.2, and the formation of an intermediate cinnamoyl-derivative of the enzyme has been demonstrated in each case (Bender, Kaiser and Zerner, J. Amer. Chem. Soc., 1961, 83, 4656). The kinetic mechanism of action of these enzymes can thus be summarized in the expression:



..... (2)

Both the enzyme-substrate complex and the acyl-enzyme are assumed to contain acidic groups which must lose a proton for reaction to proceed. The pK_a values of these

groups are not necessarily identical. Indeed, if an imidazole group is implicated, hydrogen-bonding between the hydroxyl group of the serine residue and imidazole could occur in the enzyme-substrate complex, but not in the acyl-enzyme. On the other hand, hydrogen-bonding could occur between the imidazolium group and the carbonyl-group in the acyl-enzyme. Consequently, the pK_a of the group in the enzyme-substrate complex would be expected to be lower than that of the group in the acyl-enzyme. Such a difference has been found in the chymotrypsin-catalysed hydrolysis of p-nitrophenyl acetate (Gutfreund and Sturtevant, Proc. Nat. Acad. Sci., 1956, 42, 719). These authors have also derived the kinetic equations which govern the three-stage mechanism outlined above. When the steady state has been established, the velocity of reaction is given by:

$$V = \frac{k_2' k_3' [S] [E]}{(k_2' + k_3') [S] + k_3' K_M} \quad \ldots\ldots\ldots\ldots (3)$$

where $$k_2' = k_2 \frac{K_1}{K_1 + [H^+]} \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

and $$k_3' = k_3 \frac{K_2}{K_2 + [H^+]} \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Experimentally an apparent rate constant $k_{3(app)}'$ and an apparent Michaelis constant $K_{M(app)}$ are obtained, and these are related to the true rate constants as follows:-

$$\frac{1}{k_{3(app)}'} = \frac{1}{k_2'} + \frac{1}{k_3'} \quad \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

$$K_{M(app)} = \frac{k_3'}{k_2 + k_3} K_M = \frac{k_3'}{k_2 + k_3} \frac{k_{-1} + k_2'}{k_1} \quad (7)$$

The determination of the effect of pH on $k_{3(app)}'$ leads to an apparent dissociation constant, $K_{(app)}$, which is a function of the dissociation constants K_1 and K_2 and the rate constants k_2 and k_3.

$$K_{(app)} = \frac{K_1 K_2 (k_2 + k_3)}{k_2 K_1 + k_3 K_2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (8)$$

When deacylation of the acyl-enzyme is rate-determining, $k_2 \gg k_3$, and, provided that K_2 does not greatly exceed K_1

$$K_{(app)} \approx K_2$$

Conversely, if the acylation of enzyme is rate-determining, $k_2 \ll k_3$, and, provided that K_1 does not greatly exceed K_2

$$K_{(app)} \approx K_1$$

In general, let $k_2 = nk_3$ and $K_1 = mK_2$.

$$\text{Hence, } K_{(app)} = \frac{K_1 (n + 1)}{mn + 1} = \frac{mK_2 (n + 1)}{mn + 1}$$

If $m > 1$, $K_1 > K_{(app)} > K_2$ and conversely, if $n < 1$, $K_2 > K_{(app)} > K_1$.

Thus, $K_{(app)}$ always lies between the two dissociation constants, but under special conditions may approximate to one of them.

We have studied the kinetics of the trypsin-catalysed hydrolysis of the methyl, ethyl, and cyclohexyl esters of α-N-toluene-p-sulphonyl-L-arginine (TAME, TAEE, and TACHE respectively). By using substrate concentrations high enough to ensure zero-order kinetics, we were able to measure $k'_{3(app)}$ over a range of temperatures and thus to determine the activation constants. The results are presented in Tables 3 and 4.

Table 3.

Rate constants at pH 8.4 for the trypsin-catalysed hydrolysis of derivatives of arginine and homoarginine.

Rate constants ($k'_{3(app)}$) are quoted with standard deviations; the number of determinations is given in parentheses.

t°C	TAME ($sec.^{-1}$)	TAEE ($sec.^{-1}$)	TACHE ($sec.^{-1}$)	BAEE ($sec.^{-1}$)	THAME ($sec.^{-1}$)
15		49.22±2.21 (4)	63.44±2.39(4)	7.086±0.174(4)	2.286 ±0.098(7)
20		67.41±2.80(4)	85.84±8.86(20)	10.48 ±0.66 (4)	3.042 ±0.108(8)
23	85.72±1.35 (4)				
25	93.00±8.73(17)	93.45±0.47(3)	108.6 ±4.0 (6)	14.38±0.62 (4)	4.041 ±0.212(8)
28	110.6 ±5.5 (6)				
30	124.7 ±6.6 (10)	123.8 ±1.7 (3)	146.7 ±8.7 (5)	21.49±1.37 (6)	5.331± 0.231(8)
37	176.8 ±7.5 (6)		183.8 ±7.7 (8)		

Table 4.

Activation constants at pH 8.4 for the trypsin-catalysed hydrolysis of derivatives of arginine and homoarginine.

Activation constants, with standard deviations, were calculated by the method of weighted least squares.

	E_a (cals./mole)	ΔH^+ (cals./mole at 25°)	ΔS^+ (cals./mole/°C at 25°)	ΔG^+ (cals./mole at 25°)
TAME	9430 ± 110	8830 ± 110	-19.8 ± 0.4	14749 ± 2
TAEE	10450 ± 320	9860 ± 320	-16.4 ± 1.1	14762 ± 2
TACHE	8700 ± 390	8110 ± 390	-22.0 ± 1.3	14679 ± 11
BAEE	12510 ± 350	11920 ± 350	-13.2 ± 1.2	15856 ± 10
THAME	9810 ± 70	9220 ± 70	-24.8 ± 0.2	16621 ± 2

It will be seen that there are small but definite differences between the values obtained using TAME, TAEE, and TACHE. There are two possible explanations. The hydrolysis of the acylated trypsin may not be rate-determining and $k'_{3(app)}$ may include contributions from both k'_2 amd k'_3. Alternatively, the mechanism is more complex than that outlined above. Bender and Glasson (J. Amer. Chem. Soc., 1960, 82, 3336) have obtained strong evidence for the presence of a site in chymotrypsin which binds water or alcohols, and it is possible that a similar site exists in trypsin. The hydrolysis of the acylated enzyme may be an intramolecular process involving a molecule of water bound at a site close to the acyl group. If the molecule of alcohol, which is formed during the acylation of trypsin by the ester substrate, competes with water for this site, the acyl-trypsin intermediates formed from the three substrates can no longer be regarded as identical.

We pointed out in the previous Final Technical Report that the entropy of activation for the hydrolysis of THAME is more negative than for the hydrolysis of TAME, suggesting a greater degree of structural organisation in the activated complex. This may reflect a deformation of the enzyme, an increased binding of solvent or a more complex distribution of charges over one of the activated complexes involved in the hydrolytic mechanism. Unfortunately, we have no information so far concerning the nature of the rate-determining step.

The kinetic constants for the trypsin-catalysed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) are close to those recorded for the related methyl ester (BAME) (Schwert and Eisenberg, J. Biol. Chem., 1949, 179, 665) after correction of the value for $\Delta S^{\ddagger}$ reported earlier (Final Technical Report, DA-91-591-EUC-1301). This supports the postulate that a common intermediate (α-N-benzoyl-L-arginyl)trypsin is formed and that deacylation of this by water is rate-determining. It also implies that ethanol and methanol are not appreciably bound in the acylated intermediate.

Determination of $k'_{3(app)}$ at various pH values and temperatures has been carried out using the TAME-trypsin system. We were able to calculate the apparent dissociation constants at the different temperatures (Table 5), $K_{(app)}$, and the apparent heat of ionisation.

Table 5.

Temperature (°C).	pK_a value	
	TAME	THAME
15	7.770 ± 0.020	
20	7.688 ± 0.044	
25	7.529 ± 0.048	6.934 ± 0.037
30	7.450 ± 0.038	
37	7.394 ± 0.048	

The apparent heat of ionisation for the TAME-trypsin system was 7070 ± 530 cals./mole, a value consistent with that for an imidazole nucleus. It is notable that the pK_a for the THAME-trypsin is considerably lower than that for the TAME-trypsin system at the same temperature. Since it is likely that $K_1 > K_2$ or $pK_1 < pK_2$, the lower apparent pK_a for the THAME-trypsin system may be an indication that the ratio k'_2/k'_3 is much lower for this system than for the TAME-trypsin system. In other words, the acylation step may be the main rate-limiting stage in the mechanism. Our results differ somewhat from those of Ronwin (Biochim. Biophys. Acta, 1959, 33, 326), but the conditions were rather different.

We next compared the effect of cholate on the kinetics of the trypsin-catalysed hydrolyses of TAME and TAEE at high substrate concentrations. Unfortunately a similar investigation using TACHE was precluded, since addition of cholate to an aqueous solution of this substrate gave a precipitate. The hydrolysis of both substrates was accelerated by cholate and the results, presented in Tables 6 and 7, show that the rates of hydrolysis, $k'_{3(app)}$, and activation constants for the two substrates become much closer for the two substrates in presence of cholate. Thus k'_2 and/or k'_3 must be increased by cholate. It is, of course, possible that one of these rate constants is decreased by cholate provided that the other is sufficiently increased to give rise to an overall increase in $k'_{3(app)}$, and an increase in the ratio k'_2/k'_3 might explain the convergence of the activation constants for TAME and TAEE in presence of cholate. This phenomenon could be also explained if cholate helps to remove the bound alcohol from the specific site on the acylated trypsin, and permit its replacement by water. Such a process would increase the rate of hydrolysis of the acylated trypsin (i.e. increase k'_3) and would explain, at least in part, the observed acceleration of the hydrolyses of TAME, TAEE, and THAME by cholate. Cholate may intervene in these reactions in other ways, however, and the relative importance of the various effects may depend on the particular substrate. Thus it will be seen (Table 7) that when TAME or TAEE are used as substrates, cholate tends to decrease $\Delta H^{\ddagger}$ and to make $\Delta S^{\ddagger}$ more negative. Conversely, in the hydrolysis of THAME, although this also is accelerated by cholate, addition of cholate increases $\Delta H^{\ddagger}$ and makes $\Delta S^{\ddagger}$ less negative. If it is assumed for the moment that deacylation of the acylated enzyme is the rate-determining step, cholate apparently affects the structure of the activated state as well as possibly influencing the binding of water or alcohol in the ground state. Such perturbations of the activated state might include (1) alteration of the helical content of the polypeptide chain, (2) alteration of the degree of solvent binding, (3) alteration of the number and distribution of charges. Alternatively, if the foregoing postulate that the TAME-trypsin and THAME-trypsin systems differ with regard

to the k'_2/k'_3 ratio is correct, the opposite effects of cholate on the apparent activation constants may reflect the differing weights of k'_2 and k'_3 in the composite rate constant $k'_{3(app)}$ for the two systems. It is interesting to note that increasing cholate concentration above 0.002 M has little further effect on the kinetics of the hydrolysis of THAME, unlike the case of TAME, where cholate continues to modify the kinetics up to a concentration of 0.006 M. The effects of cholate on the hydrolysis of the above substrates by trypsin is not due to general-base catalysis, since acetate ion was inactive.

Table 6.

Rate constants at pH 8.4 for the trypsin-catalysed hydrolysis of derivatives of arginine and homoarginine in presence of cholate.

Rate constants ($k'_{3(app)}$) are quoted with standard deviations; the number of determinations is given in parentheses.

t°C	Cholate (mM)	TAME	TAEE	THAME	BAME
15	0	57.98 ± 2.08 (10)	49.22 ± 2.21 (4)	2.286 ± 0.098 (7)	7.086 ± 0.174 (4)
15	2	69.20 ± 3.71 (4)	71.27 ± 2.39 (4)	2.453 ± 0.105 (8)	7.072 ± 0.068 (3)
15	4	81.66 ± 2.97 (4)	89.17 ± 7.11 (4)	2.589 ± 0.062 (4)	6.406 ± 0.169 (4)
15	6	92.33 ± 3.39 (4)	97.61 ± 2.69 (4)	2.498 ± 0.074 (4)	6.127 ± 0.291 (4)
20	0	79.09 ± 2.21 (8)	67.41 ± 2.80 (4)	3.042 ± 0.108 (8)	10.48 ± 0.66 (4)
20	2	90.21 ± 2.89 (4)	98.11 ± 4.69 (4)	3.406 ± 0.065 (7)	9.767 ± 0.310 (4)
20	4	104.5 ± 8.1 (4)	117.4 ± 5.9 (4)	3.696 ± 0.228 (4)	9.528 ± 0.369 (4)
20	6	121.5 ± 7.1 (4)	135.4 ± 3.3 (3)	3.672 ± 0.107 (4)	9.060 ± 0.126 (4)
23	0	85.72 ± 1.35 (4)			
23	2	114.7 ± 3.3 (4)			
23	4	133.0 ± 3.6 (4)			
23	6	145.3 ± 1.6 (4)			
25	0	93.00 ± 8.73 (17)	93.45 ± 0.47 (3)	4.041 ± 0.212 (8)	14.38 ± 0.62 (4)
25	2	110.8 ± 4.4 (15)	124.6 ± 0.8 (4)	4.411 ± 0.194 (8)	14.16 ± 1.27 (4)
25	4	129.1 ± 7.7 (15)	152.3 ± 9.8 (4)	4.766 ± 0.067 (4)	13.38 ± 0.37 (4)
25	6	144.3 ± 6.9 (15)	166.9 ± 1.4 (3)	4.874 ± 0.135 (4)	12.75 ± 0.46 (4)
28	0	110.6 ± 5.5 (6)			
28	2	141.4 ± 1.5 (5)			
28	4	166.4 ± 5.4 (6)			
28	6	183.8 ± 5.4 (6)			
30	0	124.7 ± 6.6 (10)	123.8 ± 1.7 (3)	5.331 ± 0.231 (8)	21.49 ± 1.37 (6)
30	2	159.2 ± 9.1 (4)	162.4 ± 4.1 (4)	6.083 ± 0.229 (8)	20.27 ± 1.60 (4)
30	4	189.5 ± 3.7 (4)	194.7 ± 12.5 (4)	6.437 ± 0.365 (4)	19.86 ± 1.26 (4)
30	6	209.0 ± 12.6 (4)	207.0 ± 4.2 (4)	6.428 ± 0.337 (4)	18.87 ± 1.04 (4)
37	0	176.8 ± 7.5 (6)			
37	2	221.7 ± 3.2 (5)			
37	4	250.4 ± 5.0 (4)			
37	6	279.4 ± 5.7 (4)			

Table 7.

Activation constants at pH 8.4 for the trypsin-catalysed hydrolysis of derivatives of arginine and homoarginine in presence of cholate.

Activation Constant	Cholate (mM)	TAME	TAEE	THAME	BAEE
E_a (cals./mole)	0	9430 ± 110	10450 ± 320	9810 ± 70	12510 ± 350
	2	9330 ± 340	9440 ± 230	10280 ± 360	11690 ± 450
	4	8940 ± 410	9010 ± 70	10400 ± 230	12750 ± 300
	6	8560 ± 120	8460 ± 580	11060 ± 610	12690 ± 370
$\Delta H^{\ddagger}$ (cals./mole at 25°C.)	0	8830 ± 110	9860 ± 320	9220 ± 70	11920 ± 350
	2	8740 ± 340	8840 ± 230	9680 ± 360	11090 ± 450
	4	8340 ± 410	8420 ± 70	9810 ± 230	12160 ± 300
	6	7970 ± 120	7860 ± 580	10470 ± 610	12100 ± 370
$\Delta S^{\ddagger}$ (cals./mole/°C at 25°C.)	0	-19.8 ± 0.4	-16.4 ± 1.1	-24.8 ± 0.2	-13.2 ± 1.2
	2	-19.7 ± 1.1	-19.3 ± 0.8	-23.0 ± 1.2	-16.1 ± 1.5
	4	-20.7 ± 1.3	-20.3 ± 0.2	-22.5 ± 0.8	-12.6 ± 1.0
	6	-21.7 ± 0.4	-22.0 ± 1.9	-20.3 ± 2.1	-12.8 ± 1.3
$\Delta G^{\ddagger}$ (cals./mole at 25°C.)	0	14749 ± 2	14762 ± 2	16621 ± 2	15856 ± 10
	2	14610 ± 8	14591 ± 2	16552 ± 7	15886 ± 10
	4	14509 ± 10	14474 ± 1	16523 ± 4	15906 ± 7
	6	14443 ± 2	14419 ± 7	16510 ± 13	15929 ± 7

The trypsin-catalysed hydrolysis of BAEE, unlike those of substrates bearing a α-N-toluene-p-sulphonamide group, was inhibited slightly by cholate. Since the available evidence indicates that acylation of trypsin by BAEE is so fast that $k'_{3(app)} = k'_3$, it now appears that cholate might accelerate the acylation step and slightly inhibit the deacylation stage.

We have found that the apparent K_M for the TAME-trypsin system is much lower than the values quoted by Martin, Golubow, and Axelrod (J. Biol. Chem., 1959, 234, 1718) and Ronwin (Biochim. Biophys. Acta, 1959, 33, 326). The former workers determined K_M at pH 8.0 and 25° in 0.1 M calcium chloride, while Ronwin used either veronal or succinate buffer at 38° and determined K_M over a range of pH values in the presence of 0.0066 M calcium chloride. We are unable to account for the lack of agreement, although the conditions were not identical; in particular, we determined K_M in the absence of buffers. At pH 8.4 and 25°, in presence of 0.001 M calcium chloride, we find that the $K_{M(app)}$ for the TAME-trypsin system is 6.48 ± 0.21 x 10^{-6} M. In presence of 0.002 M cholate, the value is 1.09 ± 0.08 x 10^{-5} M. The value of $K_{M(app)}$ is sensitive to alterations in the values of all four rate constants, k_1, k_{-1}, k'_2 k'_3. Reference to equation 7 shows that an increase in k'_3 would increase

$K_{M(app)}$, while an increase in k_1 or k_{-1} would decrease $K_{M(app)}$. The effect of alterations to k'_2 depend on the relative magnitude of k_{-1} and k'_3. If $k_{-1} > k'_3$, an increase in k'_2 would decrease $K_{M(app)}$. Theoretically, if $k_{-1} < k'_3$, the converse would be true, but this would be most unusual for an enzyme-catalysed reaction. It is not possible to determine from the present data which rate constants are affected by cholate in the TAME-trypsin system.

Values of $K_{M(app)}$ for the TAME-trypsin system under various conditions are recorded in Table 8.

Table 8.

$K_{M(app)}$ values for the TAME-trypsin system at 25°.

(Values of $K_{M(app)}$ are quoted with standard deviations; the number of complete determinations involving 6 - 8 runs is given in parentheses).

pH	Cholate conc'n.(mM)	10^4 $K_{M(app)}$ (moles/litre)
7.0	0	2.391 ± 0.070 (3)
8.4	0	3.320 ± 0.017 (4)
8.4	2	2.543 ± 0.288 (1)
8.4	6	2.243 ± 0.058 (2)

It will be seen that $K_{M(app)}$ increases with pH over the range 7.0 - 8.4. Since K_1, the dissociation constant of the group in the Michaelis complex is higher than K_2, it is likely that k'_3 increases considerably over this range, while k'_2 is much less sensitive. The increase in $K_{M(app)}$ with increasing pH is probably attributable, therefore, to the increase in k'_3. The decrease in $K_{M(app)}$ in presence of cholate, on the other hand, is probably due to an increase in k'_2. The value of k'_3 probably does not alter appreciably, as suggested by the foregoing work, but a small change in either direction is possible.

We were unable to study the trypsin-catalysed hydrolysis of α-*N*-toluene-*p*-sulphonyl-*L*-ornithine methyl ester (TOME) at pH 8.4, in view of the lactonization of the substrate, which is catalysed by hydroxyl ions (Part I). Trypsin was found to catalyse the hydrolysis of TOME at pH 7.0, however, with little concomitant lactonization. The $K_{M(app)}$ must be very high, since there was no departure from first-order kinetics with respect to substrate even when the substrate concentration was as high as 5×10^{-3}M. Pseudo-first-order rate constants are recorded in Table 9.

Table 9.

Pseudo-first-order rate constants for the TOME-trypsin system at pH 7.0.

(Rate constants are quoted with standard deviations; the number of determinations is given in parentheses).

t°C	k (sec.$^{-1}$/mole trypsin/litre).	
	No cholate	2 mM cholate
15	66.69 ± 1.71 (4)	89.06 ± 2.18 (4)
20	97.83 ± 3.06 (4)	123.08 ± 2.57 (6)
25	133.02 ± 3.48 (4)	169.58 ± 2.78 (4)
30	193.39 ± 2.61 (6)	241.06 ±11.90 (6)

In the presence of 0.002 M cholate, the apparent energy of activation is decreased from 12280 ± 290 cals./mole. to 11180 ± 260 cals./mole. It is not possible to decide from the present data which step of the enzymic mechanism is affected by cholate. The hydrolysis of TOME by trypsin does not appear to be completely stereospecific. Using the DL ester, we found that the rate of alkali uptake in presence of trypsin was greater than when the L-ester of half the molar concentration was used as substrate. Moreover, when the DL-ester was used as substrate, alkali uptake continued beyond the value representing 50% hydrolysis. This was shown not to be due to lactamization, since a sample was hydrolysed with trypsin until 74.8% of the theoretical amount of alkali required for complete hydrolysis had been used. At that point a Sørensen titration indicated that only 8.6% of the total amount of ester had lactamized. Finally, although good first-order plots could be obtained from the hydrolysis of the L-ester in presence of trypsin, similar analysis of kinetic runs with the DL-ester gave curves, indicating that at least two reactions with different rate constants were proceeding simultaneously. It is possible that the loss of complete stereospecificity is due to the shortness of the side-chain of ornithine compared with arginine and lysine. Attachment of TOME to a specific site on the enzyme molecule by the protonated amino group may be weakened. Further shortening of the side-chain might preclude any attachment to the specific site and this presumably explains the failure of trypsin to catalyse the hydrolysis of ATEE.

PART III. STUDIES OF THE KINETICS AND MECHANISM OF THROMBIN-CATALYSED HYDROLYSES OF SYNTHETIC SUBSTRATES

Thrombin is a proteolytic enzyme which is responsible for the conversion of fibrinogen into fibrin during blood-clotting; it also displays esterase activity towards substrates like TAME. Inhibition of thrombin by di-isopropyl phosphoro-fluoridate, followed by partial hydrolysis, has revealed that this enzyme, like trypsin, chymotrypsin, elastase, and cholinesterase, has the amino-acid sequence - gly.asp.ser.gly - at the active centre. Unfortunately, thrombin has not been purified as completely as trypsin or chymotrypsin, and it is necessary to define a suitable unit of activity. The literature abounds with different units for expressing thrombin activity, and we have selected that due to Ehrenpreis and Scheraga (J. Biol. Chem., 1957, 227, 1043) as the most suitable for our purpose. One thrombin unit is defined as that amount of enzyme required to liberate 0.1 μmole of acid per minute from 1 ml. of 0.01 M TAME at pH 8 in 0.15 M KCl at 25°. We were able to confirm the observation of Ehrenpreis and Scheraga that thrombin solutions at pH 7 and 0°C retain their esterase activity for at least nine days. On the other hand Ronwin (Canad. J. Biochem. Physiol., 1957, 35, 743) reports that thrombin, dissolved in glycerol-water (1 : 1) or isotonic saline, becomes more active as an esterase during storage at 0-4°. Using BAEE (5×10^{-3}M) as substrate, we found that zero-order kinetics were obeyed and the velocity was proportional to the enzyme concentration.

The thrombin-catalysed hydrolysis of TAME is markedly dependent on ionic strength. Ronwin (Canad. J. Biochem. Physiol., 1957, 35, 743) has reported that increase of ionic strength inhibited this reaction, but since he used buffers as well, the results are difficult to interpret. Our results (Table 10) were obtained in the absence of buffers, and it can be clearly seen that k_3' (app) decreases asymptotically with increasing strength to a limiting value which depends on the cation. Our results do not afford a linear relationship between log k_3' and $\sqrt{\mu}$, where μ is the ionic strength, as might have been expected. Scheraga and Ehrenpreis (Proc. 4th Intern. Congr. Biochemistry, Vienna, Pergamon Press, Vol. X, p.212) have briefly reported that sodium chloride inhibits the hydrolysis of TAME by thrombin as the ionic strength is increased. Their results qualitatively resemble ours and afford no linear relationship between log k_3' and $\sqrt{\mu}$. A possible explanation for the complex connexion between log k_3' and μ may be the presence of more than one esterase activity in the thrombin preparations.

Table 10.

Effect of ionic strength on $k'_{3\,(app)}$ for the thrombin-catalysed hydrolysis of TAME at pH 8.4 and 25°C.

$k'_{3\,(app)}$ values are quoted with standard deviations; the number of determinations is given in parentheses.

Conc'n. of salt (M)	$10^5 k'_{3\,(app)}$ (moles/litre/min./Scheraga unit/litre).	
	NaCl	KCl
0.00	18.071 ± 0.076 (5)	18.071 ± 0.076 (5)
0.05	9.082 ± 0.663 (3)	13.104 ± 0.381 (3)
0.10	6.754 ± 0.431 (4)	12.361 ± 0.238 (2)
0.20	5.114 ± 0.808 (2)	10.802 ± 0.751 (2)
0.30	4.289 ± 0.104 (2)	8.832 ± 0.125 (2)
0.40	3.880 ± 0.287 (2)	8.809 ± 0.166 (2)

Surprisingly, the rate of hydrolysis of BAEE by thrombin was insensitive to the ionic strength when sodium or potassium chloride was added to the system. A possible explanation of the inhibition of hydrolysis of TAME by thrombin with increasing ionic strength is the existence of a mechanism which involves the approach of ions of unlike charges. Since TAME has a positive charge on the guanidine group, it might be argued that a negative charge exists at the active centre of thrombin. Such a charge does exist on the β-CO_2^- group of the aspartyl residue, adjacent to the serine residue which is phosphorylated by di-isopropyl phosphorofluoridate. This explanation is weakened, however, by the insensitivity of the BAEE-thrombin and TAME-trypsin systems to change of ionic strength. On the other hand, it is possible, as outlined below, that the acylation step controlled by k'_2 contributes to $k'_{3\,(app)}$ in the TAME-thrombin system, but not in the BAEE-system. In this event, the acylation step only could be sensitive to change of ionic strength, in which case, it could be an indication that a negatively charged group is the nucleophile which attacks the carbonyl-carbon atom in the substrate. Further evidence must be obtained before this point can be established. It is puzzling that the TAME-trypsin system is insensitive to change of ionic strength when other evidence indicates that the acylation step is partially responsible for the observed value of $k'_{3\,(app)}$. It is of interest to recall that the velocities of certain hydrolyses catalysed by chymotrypsin and ficin are increased as the ionic strength is increased (Shine and Niemann, J. Amer. Chem. Soc., 1955, 77, 4275; Martin and Niemann, J. Amer. Chem. Soc., 1958, 80, 1481; Hammond and Gutfreund, Biochem. J., 1959, 72, 349).

We have determined k_3' (app) for the hydrolysis of a range of esters in presence of thrombin at pH 8.4 and 25°C. These include TAME, TAEE, TACHE, BAEE, BAME, α-N-toluene-p-sulphonyl-L-arginine n-propyl ester (TAPE), and α-N-toluene-p-sulphonyl-L-lysine methyl ester (TLME). The results are presented in Table 11.

Table 11.

k_3' (app) for the hydrolysis of various substrates by thrombin at pH 8.4 and 25°C in 0.1 M sodium chloride solution.

Values of k_3' (app) are quoted with standard deviations; the number of determinations is given in parentheses.

Substrate	$10^5 k_3'$ app) (moles/litre/min./Scheraga unit/litre).
TAME	6.754 ± 0.431 (4)
TAEE	2.988 ± 0.051 (10)
TAPE	2.611 ± 0.056 (6)
TACHE	6.538 ± 0.088 (6)
TLME	10.430 ± 0.601 (6)
BAME	3.233 ± 0.071 (5)
BAEE	3.246 ± 0.013 (15)

It will be noticed that TACHE is hydrolysed considerably more rapidly than TAEE or TAPE, and it is interesting that this order is the opposite of that expected in the non-enzymic alkaline hydrolysis of esters. As was found in the case of trypsin, esters containing a α-N-toluene-p-sulphonamide group do not hydrolyse at the same rate. Hence, we may conclude that for these substrates, deacylation of an acyl-thrombin is not uniquely rate-determining; the acylation step governed by k_2' makes a significant contribution to k_3' (app). On the other hand BAME and BAEE are hydrolysed by thrombin at indistinguishable rates, and it may be concluded that deacylation is rate-determining, i.e. k_3' (app) = k_3'. The foregoing observations are thus similar to but rather more striking than those found using trypsin. One further point may be noted; TLME and TAME are hydrolysed at about the same rate by thrombin. The distance between the charged group in the side-chain and the ester-carbonyl group is almost identical for the two substrates when the molecules are in their energetically most favoured conformations. We have indicated in the studies on trypsin that the length of the side-chain of the substrate is important in determining specificity, and the same seems to be true in the case of thrombin. In preliminary experiments, we have found that TOME is slowly hydrolysed by thrombin,

and the apparent K_M is high, as in the case of trypsin. THAME, on the other hand, is not appreciably hydrolysed by thrombin, and in this respect contrasts sharply with trypsin.

In the previous Final Technical Report we recorded the results of a limited study of the effect of temperature variation on k'_3 (app) for the TAME-thrombin system. The results available at that time indicated that an Arrhenius type of plot was linear. More extensive measurements using both TAME and BAEE as substrates have since shown that plots of log k'_3 (app) against 1/T have a marked curvature (Tables 12 and 13, Figs. 1 and 2). We are thus unable to calculate activation constants for thrombin-catalysed reactions. It is likely that this abnormal behaviour results from the presence of more than one type of esterase activity in the thrombin preparation. This is substantiated by the very recent isolation by Marciniak and Seegers (Canad. J. Biochem. Physiol., 1962, 40, 597) of two fractions possessing esterase activity from highly purified thrombin. Ronwin (Canad. J. Biochem. Physiol., 1957, 35, 743) found that zero-order kinetics in the TAME-thrombin system were not obeyed at 38° when small concentrations of enzyme were employed, but normal behaviour was found in experiments when relatively large amounts of thrombin were used. Neither we nor Sherry and Troll (J. Biol. Chem., 1954, 208, 95) have observed this phenomenon.

Table 12.

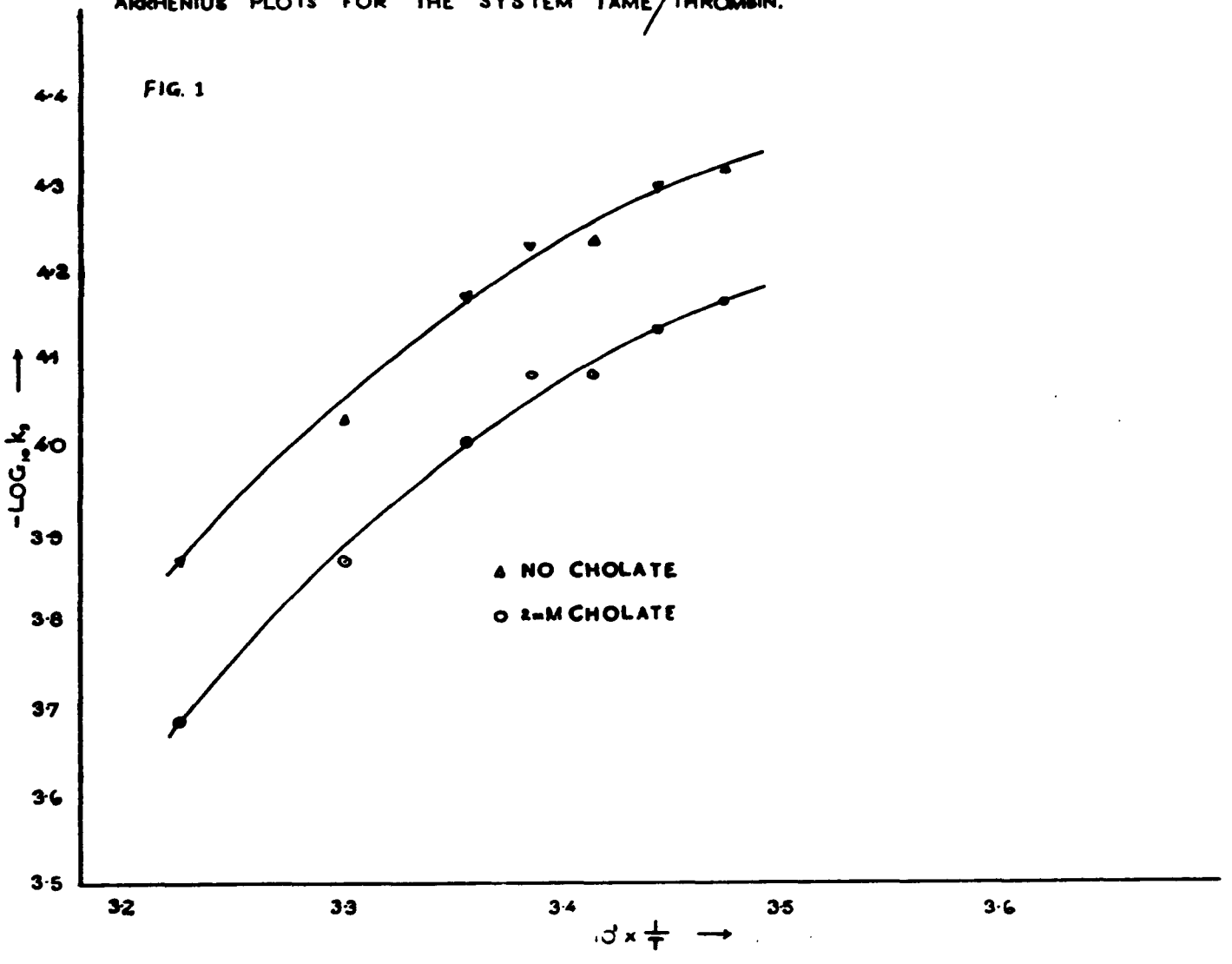
Effect of temperature on k'_3 (app) for the TAME-thrombin system at pH 8.4 alone and in the presence of cholate.

Values of k'_3 (app) are quoted with standard deviations; the number of determinations is given in parentheses.

Temperature t°C	$10^5 k'_3$ (app) (moles/litre/min./Scheraga unit/litre) No cholate	2 mM cholate
15	4.826 ± 0.197 (3)	6.786 ± 0.252 (3)
17.5	5.058 ± 0.048 (4)	7.289 ± 0.095 (4)
20	5.869 ± 0.031 (4)	8.231 ± 0.353 (4)
22.5	5.965 ± 0.124 (4)	8.207 ± 0.157 (4)
25	6.754 ± 0.431 (4)	9.875 ± 0.337 (5)
30	9.486 ± 0.517 (4)	13.533 ± 0.020 (4)
37	13.499 ± 0.015 (4)	20.512 ± 0.056 (4)

ARRHENIUS PLOTS FOR THE SYSTEM TAME/THROMBIN.

FIG. 1



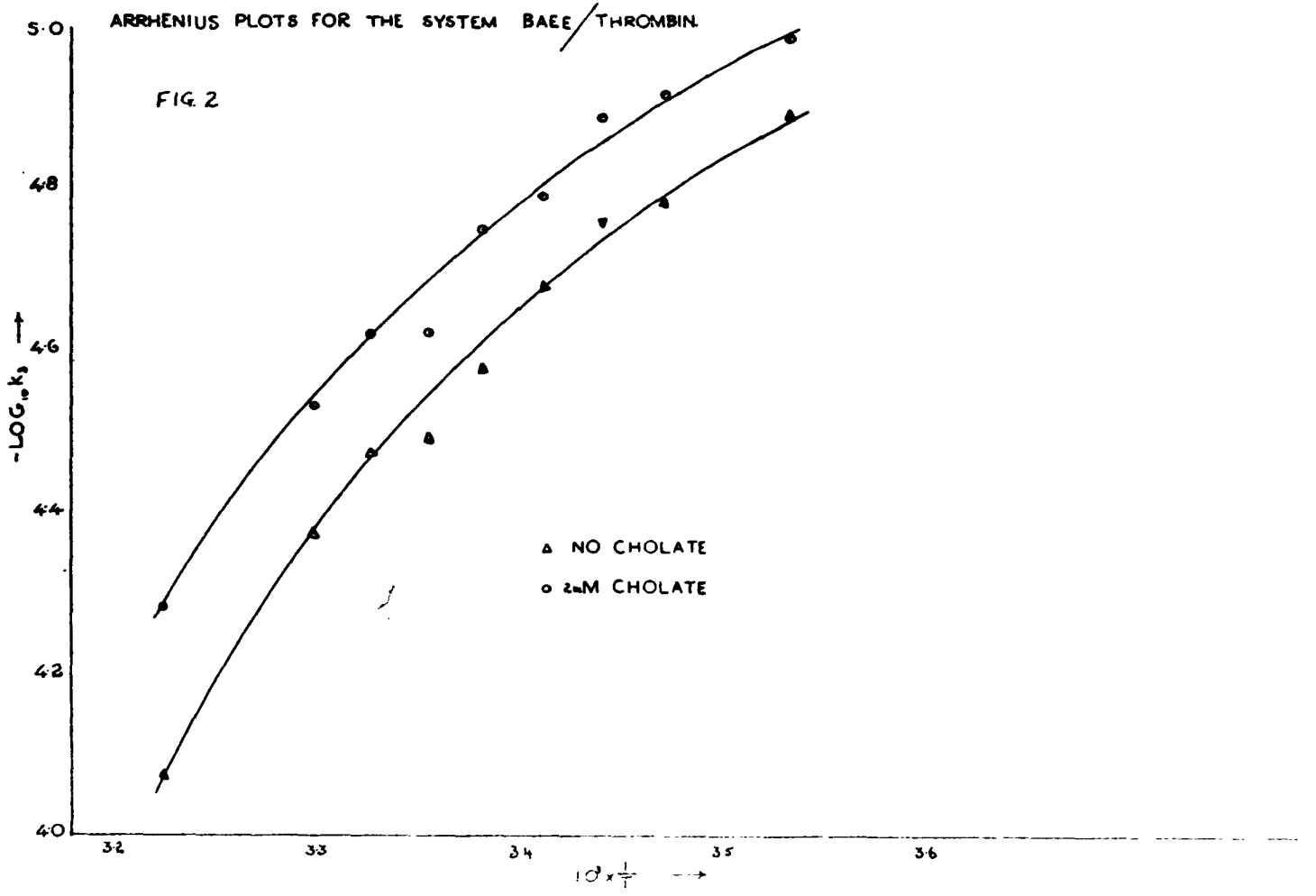

ARRHENIUS PLOTS FOR THE SYSTEM BAEE / THROMBIN.
FIG. 2
5·0
4·8
4·6
4·4
4·2
4·0
$-\log_{10} k_2$
3·2
3·3
3·4
3·5
3·6
$10^3 \times \frac{1}{T}$
△ NO CHOLATE
○ 2mM CHOLATE

Table 13.

Effect of temperature on k'_3 (app) for the BAEE-thrombin system at pH 8.4 alone and in the presence of cholate.

Values of k'_3 (app) are quoted with standard deviations; the number of determinations is given in parentheses.

Temperature	$10^5 k'_3$ (app) (moles/litre/min./Scherage unit/litre)			
t°C	No cholate	2mM cholate	4mM cholate	6mM cholate
10	1.283 ± 0.023 (4)	1.043 ± 0.044 (4)		
15	1.627 ± 0.037 (3)	1.197 ± 0.051 (4)	1.116 ± 0.055 (3)	1.047 ± 0.053 (3)
17.5	1.695 ± 0.017 (4)	1.279 ± 0.017 (4)		
20	2.101 ± 0.061 (4)	1.703 ± 0.011 (4)		
22.5	2.754 ± 0.013 (4)	1.773 ± 0.065 (3)		
25	3.246 ± 0.013(15)	2.370 ± 0.088 (3)	1.798 ± 0.059 (3)	1.603 ± 0.069 (3)
27.5	3.377 ± 0.025 (4)	2.388 ± 0.096 (3)	1.826 ± 0.067 (3)	1.690 ± 0.108 (3)
30	4.212 ± 0.051 (3)	2.926 ± 0.091 (5)	2.457 ± 0.093 (5)	2.217 ± 0.065 (5)
37	8.937 ± 0.021 (4)	5.236 ± 0.011 (3)	4.105 ± 0.020 (4)	3.519 ± 0.016 (4)

Ronwin (Biochim. Biophys. Acta, 1959, 33, 326) has studied the effect of pH-variation on k'_3 (app) for the TAME-thrombin system and has reported that there are two dissociating groups in the ES complex, and three in the free enzyme. In contrast, we detected only one dissociable group, $pK_{(app)} = 6.46 \pm 0.06$, in the TAME-trypsin system by measuring k'_3 (app) at various pH values at high substrate concentration. This could be an imidazole group, but it is likely that the observed pK_a lies between those of the dissociable group on the ES complex and that on the acylated enzyme, since it appears from the results described above that acylation is partly rate-determining.

The thrombin-catalysed hydrolyses of substrates such as TAME, TAEE, TLME, and TOME, which contain a α-*N*-toluene-*p*-sulphonamide group are accelerated by cholate and even more by glycocholate. The thrombin-catalysed hydrolysis of BAEE, on the other hand, is markedly inhibited by both cholate and glycocholate. The inhibition of this system is more pronounced than in the case of the trypsin-catalysed hydrolysis of BAEE. The kinetic modification by bile salts of both trypsin- and thrombin-catalysed reactions appears to operate by a similar mechanism; acylation is accelerated while deacylation is retarded. The results are recorded in Tables 12, 13, and 14. There is a quantitative difference between the effects of cholate and glycocholate. The former, from its effect on the hydrolysis of BAEE, appears to be the more powerful inhibitor of the deacylation step. Glycocholate, on the other hand, is the more effective accelerator of the hydrolysis of TAME. This is

consonant with the postulates that both k_2' and k_3' contribute to the overall rate of hydrolysis of TAME and that bile salts accelerate the acylation step governed by k_2' and inhibit the deacylation step governed by k_3'.

Table 14.

The effect of cholate and glycocholate on $k'_{3(app)}$ for thrombin-catalysed hydrolyses of derivatives of arginine and lysine at pH 8.4 and 25°.

Values of $k'_{3(app)}$ are quoted with standard deviations; the number of determinations is given in parentheses.

Bile Salt	Conc'n (mM)	$10^5 k'_{3(app)}$ (moles/litre/min./Scheraga unit/litre). TAME	TAEE	TAME	BAEE
None	0	6.754 ± 0.431(4)	2.988 ± 0.051(10)	10.430 ± 0.60(6)	3.246 ± 0.013(15)
Cholate	2	9.875 ± 0.337(5)	4.539 ± 0.062 (3)	14.17 ± 0.60(6)	2.370 ± 0.088 (3)
	4			14.94 ± 0.16(3)	1.798 ± 0.059 (3)
	6			15.96 ± 0.63(3)	1.603 ± 0.069 (3)
Glycocholate	2	13.98 ± 0.22 (5)		15.29 ± 0.30(4)	2.326 ± 0.025 (4)
	4	15.78 ± 0.49 (6)		17.80 ± 0.46(3)	2.202 ± 0.029 (4)
	6	16.09 ± 0.19 (4)		19.29 ± 0.27(4)	1.965 ± 0.140 (3)

We have also determined the apparent K_M for the BAEE- and TAME-thrombin systems alone and in the presence of cholate and glycocholate. The values of $K_{M(app)}$ in the absence of bile salts were sufficiently high to be determined by the orthodox technique. In presence of bile salts, however, the values of $K_{M(app)}$ were depressed to an extent that made it necessary to measure velocities during the course of single runs by the process of statistically fitting orthogonal polynomials to the experimental pH-stat traces and differentiating the polynomials at various stages during the reactions. This method can only be used if the products of hydrolysis are not inhibitory. We showed in separate experiments that addition of α-N-toluene-p-sulphonyl-L-arginine to the TAME-thrombin system or of α-N-benzoyl-L-arginine to the BAEE thrombin system did not produce any detectable alteration in velocity. The method is liable to fairly considerable error, but the effect of bile salts is sufficiently pronounced for certain trends to be seen. The results are presented in Table 15.

Table 15.

$K_{M(app)}$ for the BAEE- and TAME-thrombin systems at pH 8.4 and 25°

Mean values of $K_{M(app)}$ are quoted with standard deviations; the number of determinations is given in parentheses.

Bile Salt	$10^5 K_{M(app)}$ (moles/litre)	
	BAEE	TAME
None	1.61 ± 0.39 (5)	3.21 ± 0.78 (7)
Cholate (2mM)	0.60 ± 0.10 (6)	0.50 ± 0.23 (6)
Glycholate (2mM)	0.74 ± 0.14 (5)	0.54 ± 0.26 (5)

The effect on $K_{M(app)}$ of alterations to k_2' and k_3' is analysed in Part II. An increase in k_2' or a decrease in k_3', both of which appear to result from addition of bile salts, lead to a decrease in $K_{M(app)}$. The observation that the $K_{M(app)}$ for the TAME-thrombin system is more sensitive than that for the BAEE-thrombin system in presence of cholate is consistent with the view that $k_2' \gg k_3'$ in the latter case.

A few experiments have been carried out to determine the effect of the non-ionic detergents Tween 20, Tween 40, and Tween 80 on thrombin-catalysed reactions. The hydrolysis of BAEE is slightly accelerated, while that of TAME is considerably accelerated. In the light of the foregoing discussion, it appears that the non-ionic detergents slightly increase k_3' and effect a considerable increase in k_2'. The results are given in Table 16.

Table 16.

Effect of non-ionic detergents on $k'_{3(app)}$ for the thrombin-catalysed hydrolysis of TAME and BAEE at pH 8.4 and 25°.

Values of $k'_{3(app)}$ are quoted with standard deviations; the number of determinations is given in parentheses.

Detergent	Concentration (% W/V)	$10^5 k'_{3(app)}$ (moles/litre/min./Scheraga unit/litre).	
		TAME	BAEE
None	0	6.754 ± 0.431 (4)	3.246 ± 0.013 (15)
Tween 20	0.3	9.422 ± 0.140 (3)	3.667 ± 0.096 (4)
	1.2	11.026 ± 0.486 (3)	
Tween 40	0.3	9.219 ± 0.331 (4)	
	1.2	9.159 ± 0.097 (4)	3.505 ± 0.062 (3)
Tween 80	0.3	11.603 ± 0.301 (5)	3.920 ± 0.116 (4)

From the foregoing work, it is evident that trypsin and thrombin closely resemble one another. Thrombin, however, does appear to be somewhat more specific (see Scheraga and Laskowski, Adv. Protein Chem., 1957, 12, 1), since it has virtually no action on THAME. The conversion of fibrinogen into fibrin is a further example of the greater specificity of thrombin compared with trypsin. Although fibrin is not a substrate for thrombin, except perhaps in presence of high enzyme concentrations (Guest and Ware, Science, 1950, 112, 21), trypsin is capable of effecting considerable degradation involving the cleavage of peptide bonds. Both thrombin and trypsin appear to catalyse the hydrolysis of synthetic substrates by a three-step mechanism, and, for a given substrate, the relative importance of each step in determining the overall kinetics is very similar. The behaviour of kinetic modifiers such as cholate appears to be qualitatively similar with both enzymes; the step governed by k_2^1 is accelerated and that controlled by k_3^1 is slightly retarded. If this theory can be confirmed by further experiments using other substrates, it is possible that such kinetic modifiers will be useful in elucidating the mechanism of enzyme-catalysed processes. It is not possible yet to assess with certainty the importance of anionic surface-active agents such as bile salts or phospholipids on biological processes such as protein digestion and blood-clotting, which depend on trypsin and thrombin. The kinetic mechanism of those reactions described in this report appears to be more complex than at first seemed to be the case. We have not had time to study the effect of cholate and glycocholate on more complex reactions such as the conversion of fibrinogen into fibrin, nor have we been able to study the kinetics of reactions catalysed by plasmin. Clearly, it would be of considerable interest to do this. We would have liked to have studied the effect of bile salts on the pre-steady state using substrates such as p-nitrophenyl acetate, but delays in obtaining commercial equipment precluded this. It is hoped, however, that the authors will be in a position to tackle some of these problems in the near future.

PERSONNEL, TIME EXPENDED, AND EXPENSES.

Mr. N. J. Baines worked on this contract from its commencement until the end of the second quarter. Mr. E. F. Curragh has worked on this contract throughout the whole period. Both men have devoted their complete attention to the problem during the time that they have been paid under the contract.

The cost of chemicals and of preparing reports has exceeded the original estimate of £79.